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IMMUNOSUPPRESSIVE AND ANTIINFLAMMATORY DRUGS

Edited by Anthony C. Allison, Kevin J. Lafferty, and Hans Fliri

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IMMUNOSUPPRESSIVE AND ANTIINFLAMMATORY DRUGS[a]

Editors and Conference Organizers
ANTHONY C. ALLISON, KEVIN J. LAFFERTY, AND HANS FLIRI

CONTENTS

Novel Immunosuppressive and Antiinflammatory Drugs: A 1993 Perspective. *By* ANTHONY C. ALLISON xi

Part I. Immunobiology of Cyclosporins, FK-506, and Rapamycin

Immunosuppressive Profile of Rapamycin. *By* SUREN N. SEHGAL 1

The Mechanism of Action of FK-506 and Cyclosporin A. *By* GREG WIEDERRECHT, ELSA LAM, SHIRLEY HUNG, MARY MARTIN, and NOLAN SIGAL 9

Calcineurin Is a Key Signaling Enzyme in T Lymphocyte Activation and the Target of the Immunosuppressive Drugs Cyclosporin A and FK506. *By* NEIL A. CLIPSTONE and GERALD R. CRABTREE 20

Rapamycin Inhibits $p34^{cdc2}$ Expression and Arrests T Lymphocyte Proliferation at the G1/S Transition. *By* W. MICHAEL FLANAGAN and GERALD R. CRABTREE 31

Proline Isomerases in Microorganisms and Small Eukaryotes. *By* CHARLES HEMENWAY and JOSEPH HEITMAN 38

Cyclosporins: Structure-Activity Relationships. *By* HANS FLIRI, GOETZ BAUMANN, ALBERT ENZ, JUERG KALLEN, MARCEL LUYTEN, VINCENT MIKOL, RAO MOVVA, VALERIE QUESNIAUX, MAX SCHREIER, MALCOLM WALKINSHAW, ROLAND WENGER, GERHARD ZENKE, and MAURO ZURINI 47

An FKBP-Rapamycin-Sensitive, Cyclin-Dependent Kinase Activity that Correlates with the FKBP-Rapamycin-Induced G1 Arrest Point in MG-63 Cells. *By* MARK W. ALBERS, ERIC J. BROWN, AKITO TANAKA, RICHARD T. WILLIAMS, FREDERICK L. HALL, and STUART L. SCHREIBER ... 54

Part II. Novel Immunosuppressive Drugs

Mechanisms of Action of Mycophenolic Acid. *By* ANTHONY C. ALLISON, W. JOSEPH KOWALSKI, CHRISTIAN D. MULLER, and ELSIE M. EUGUI 63

[a] This volume is the result of a conference entitled Immunosuppressive and Antiinflammatory Drugs held on April 12–15, 1993 in Orlando, Florida and sponsored by the New York Academy of Sciences.

Human Type I and II IMP Dehydrogenases as Drug Targets. *By* YUTAKA NATSUMEDA and STEPHEN F. CARR 88

Immunosuppression by Discodermolide. *By* ROSS E. LONGLEY, SARATH P. GUNASEKERA, DENISE FAHERTY, JOHN MCLANE, and FRANCIS DUMONT.. 94

Brequinar Sodium, Mycophenolic Acid, and Cyclosporin A Inhibit Different Stages of IL-4- or IL-13-Induced Human IgG4 and IgE Production *In Vitro*. *By* CHIA-CHUN J. CHANG, GREGORIO AVERSA, JUHA PUNNONEN, HANS YSSEL, and JAN E. DE VRIES 108

Deoxyspergualin: Mechanism of Action Studies of a Novel Immunosuppressive Drug. *By* MARK A. TEPPER 123

Part III. Novel Antiinflammatory Strategies

The IL-1β Converting Enzyme as a Therapeutic Target. *By* DOUGLAS K. MILLER, JIMMY R. CALAYCAY, KEVIN T. CHAPMAN, ANDREW D. HOWARD, MATTHEW J. KOSTURA, SUSAN M. MOLINEAUX, and NANCY A. THORNBERRY 133

Bicyclic Imidazoles as a Novel Class of Cytokine Biosynthesis Inhibitors. *By* J. C. LEE, A. M. BADGER, D. E. GRISWOLD, D. DUNNINGTON, A. TRUNEH, B. VOTTA, J. R. WHITE, P. R. YOUNG, and P. E. BENDER..... 149

Coordinate Inhibition by Some Antioxidants of TNFα, IL-1β, and IL-6 Production by Human Peripheral Blood Mononuclear Cells. *By* ELSIE M. EUGUI, BARBARA DELUSTRO, SUSSAN ROUHAFZA, ROBERT WILHELM, and ANTHONY C. ALLISON 171

Mycophenolic Acid and Some Antioxidants Induce Differentiation of Monocytic Lineage Cells and Augment Production of the IL-1 Receptor Antagonist. *By* RUTH V. WATERS, DONNA WEBSTER, and ANTHONY C. ALLISON 185

Cycloxygenase Gene Expression in Inflammation and Angiogenesis. *By* TIMOTHY HLA, ARI RISTIMÄKI, SUSAN APPLEBY, and JAVIER G. BARRIOCANAL 197

Preclinical and Clinical Activity of Zileuton and A-78773. *By* R. L. BELL, C. LANNI, P. E. MALO, D. W. BROOKS, A. O. STEWART, R. HANSEN, P. RUBIN, and G. W. CARTER 205

Part IV. Immunosuppressive Drugs for Transplantation

Use of Brequinar Sodium for Transplantation. *By* DONALD V. CRAMER, FRANCES A. CHAPMAN, and LEONARD MAKOWKA 216

Cytidine Potentiates the Inhibitory Effect of Brequinar Sodium on Xeno-MLR, Antibody Production, and Concordant Hamster to Rat Cardiac Xenograft Survival. *By* JACKY WOO, LUIS A. VALDIVIA, FAN PAN, SUSANNA CELLI, JOHN J. FUNG, and ANGUS W. THOMSON 227

Efficacy of FK506 in Renal Transplantation. *By* SHIRO TAKAHARA 235

Incidence of $CD4^+$ IL-2Rα^+ and $CD4^+$ T-Cells in Progressive Multiple Sclerosis and the Influence of Short-Term (3 Months) FK 506 Therapy. *By* ANGUS W. THOMSON, JACKY WOO, BONNIE LEMSTER, WILLIAM IRISH, LINDA L. HUANG, PATRICIA B. CARROLL, HORATIO R. RILO, KAREEM ABU-ELMAGD, and BENJAMIN EIDELMAN 245

FK 506 Inhibits Cytokine Gene and Adhesion Molecule Expression in Psoriatic Skin Lesions. *By* B. LEMSTER, H. R. RILO, P. B. CARROLL, M. A. NALESNIK, and A. W. THOMSON 250

Inhibitory Effect of FK 506 on Autoimmune Thyroid Disease in the PVG Rat. *By* K. TAMURA, J. WOO, N. MURASE, M. NALESNIK, and A. W. THOMSON 257

Deoxyspergualin: Mode of Action and Clinical Trials. *By* SEIICHI SUZUKI 263

Combination of Immunosuppressive Drugs for Organ Transplantation. *By* TAKENORI OCHIAI, YOSHIO GUNJI, MATSUO NAGATA, and KAICHI ISONO .. 270

Immunosuppressant Combinations in Primate Cardiac Xenografts: A Review. *By* ROBERT P. MCMANUS, DANIEL P. O'HAIR, RICHARD KOMOROWSKI, and J. PAUL SCOTT 281

Part V. Clinical Applications of Immunosuppressive and Antiinflammatory Drugs

Optimizing Combination Chemotherapy for Rheumatoid Arthritis. *By* DANIEL E. FURST 285

Effects of Nonsteroidal Antiinflammatory Drugs on Bone Loss in Chronic Inflammatory Disease. *By* MARJORIE K. JEFFCOAT, MICHAEL S. REDDY, LARRY W. MORELAND, and WILLIAM J. KOOPMAN 292

Cyclooxygenase Inhibitors Vary Widely in Potency for Preventing Cytokine-Induced Bone Resorption. *By* ANTHONY C. ALLISON, RUEI-CHEN CHIN, and YVONNE CHENG 303

Treatment of Autoimmune Uveitis. *By* SCOTT M. WHITCUP and ROBERT B. NUSSENBLATT 307

Immunosuppressive Treatment of Chronic Liver Disease. *By* E. ANTHONY JONES and NORA VALERIA BERGASA 319

Part VI. Treatment of Insulin-Dependent Diabetes Mellitus

Mycophenolate Mofetil Can Prevent the Developmentof Diabetes in BB Rats. *By* LIMING HAO, SIU-MEI CHAN, and KEVIN J. LAFFERTY 328

The Use of Nicotinamide in the Prevention of Type 1 Diabetes. *By* R. B. ELLIOTT, C. C. PILCHER, A. STEWART, D. FERGUSSON, and M. A. MCGREGOR 333

Early Diagnosis and Specific Treatment of Insulin-Dependent Diabetes. *By* NOEL MACLAREN, ANDREW MUIR, JANET SILVERSTEIN, YAO-HUA SONG, JIN XIONG SHE, JEFFREY KRISCHER, MARK ATKINSON, and DESMOND SCHATZ 342

Lessons Learned from Use of Cyclosporine for Insulin-Dependent Diabetes Mellitus: The Case for Immunotherapy for Insulin-Dependent Diabetics Having Residual Insulin Secretion.. *By* JEFFREY L. MAHON, JOHN DUPRE, and CALVIN R. STILLER 351

Strategies in Immunotherapy of Insulin-Dependent Diabetes Mellitus. *By* JEAN-FRANÇOIS BACH 364

New Strategies for Clinical Islet Transplantation: The Role of Purified Grafts and Immunosuppressive Therapy. *By* PAUL F. GORES, DIXON B. KAUFMAN, and DAVID E. R. SUTHERLAND 377

Poster Papers

A Powerful Antiinflammatory Therapy Is Effective in the Treatment of Idiopathic Thrombocytopenia. *By* ADRIANA MENICHELLI and DOMENICO DEL PRINCIPE 390

Interaction of Diltiazem and Rapamycin on Survival of Rat Cardiac Allografts. *By* LOUIS DUMONT, HUIFANG CHEN, DASHENG XU, and PIERRE DALOZE 392

Antioxidants Inhibit Activation of Transcription Factors and Cytokine Gene Transcription in Monocytes. *By* MARIOLA ILNICKA, SIMON W. LEE, and ELSIE M. EUGUI 396

Hydrogen Peroxide Primes Promonocytic U937 Cells to Produce IL-1β. *By* SIMON W. LEE and MARIOLA ILNICKA 399

Structure-Function Analysis of Integrin-Mediated Cell Functions by Novel Nonpeptidic Conformation Mimetics of the Arg-Gly-Asp and Leu-Asp-Val Amino Acid Sequences. *By* OFER LIDER, RAMI HERSHKOVIZ, RONEN ALON, and NOAM GREENSPOON 402

Effects of Cyclosporine-A and Cyclosporine-G on ADP-Stimulated Aggregation of Human Platelets. *By* MARIANA S. MARKELL, JULIO FERNANDEZ, ULHAS P. NAIK, YIGAL EHRLICH, and ELIZABETH KORNECKI 404

Relationship of Ionized Magnesium and Cyclosporine Level in Renal Transplant Recipients. *By* MARIANA S. MARKEL, BELLA T. ALTURA, YVONNE SARN, RANDALL BARBOUR, ELI A. FRIEDMAN, and BURTON M. ALTURA 408

Studies on the Interaction of the Immunosuppressant 15-Deoxyspergualin with Heat Shock Proteins. *By* STEVEN G. NADLER, JEFFREY CLEAVELAND, MARK A. TEPPER, CHRISTOPHER WALSH, and KARI NADEAU 412

4-Thiazolidinones, Potent Antioxidants, as Antiinflammatory Agents. *By* J. A. PANETTA, J. K. SHADLE, M. L. PHILLIPS, D. N. BENSLAY, AND P. P. K. HO 415

Effects of Methylprednisolone Administration on Lymphocyte LECAM-1, CD44, and LFA-1 Expression: Implications for Steroid-Induced Lymphopenia. *By* ROBERT SACKSTEIN 416

CMT/NSAID Combination Increases Bone CMT Uptake and Inhibits Bone Resorption. *By* N. RAMAMURTHY, M. LEUNG, S. MOAK, R. GREENWALD, and L. GOLUB 420

Index of Contributors 423

The IL-1β Converting Enzyme as a Therapeutic Target

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INTRODUCTION

Interleukin-1β (IL-1β) and interleukin-1α (IL-1α) are proinflammatory cytokines that promote leukocyte infiltration, prostaglandin synthesis, joint swelling, and tissue destruction.[1-8] IL-1α and IL-1β are members of a family of cytokines that also includes the IL-1 receptor antagonist protein (IL-1RA; see FIGURE 1), all of which are synthesized most prominently by monocytic cells. In contrast to the agonist activity of IL-1α and IL-1β,[9] IL-1RA is a strict antagonist on IL-1 receptors. It is synthesized on membrane-bound polysomes and exported via the classical endoplasmic reticulam (ER)/Golgi route where it becomes glycosylated. IL-1α and IL-1β, on the other hand, both lack leader sequences and are found in cytoplasm.[10-14] IL-1β is released from cells following stimulation, and it is the major agonist form of IL-1 found in biological fluids during diseased states.[15,16] In contrast, IL-1α remains largely intracellular in spite of its synthesis at significant levels.[13,17] IL-1RA is also released from stimulated cells, but its appearance in blood is delayed relative to that of IL-1β. Because IL-1RA is produced at about 100-fold higher concentrations than IL-1β, it may serve to decrease IL-1β activity.[18]

The importance of IL-1 as a target for antiinflammatory therapy is shown by the efficacy of IL-1RA, soluble IL-1R, and antiIL-1 receptor monoclonal antibodies in several animal models of human disease.[19-23] For example, PMN infiltration, swelling, and tissue necrosis were reduced in a rabbit model of inflammatory bowel disease by IL-1RA. Mortality was drastically reduced in murine graft vs host disease with the use of IL-1RA. Truncated soluble IL-1 receptors showed efficacy in reducing the swelling in cat adjuvant arthritis and blocking allograft rejection in mice.[24] Monoclonals against the IL-1 receptor have also been shown to block PMN extravasation and acute phase protein synthesis in mice.[25]

The importance of IL-1β as the primary form of IL-1 responsible *in vivo* has been confirmed recently by the discovery of pox virus proteins that are specific for IL-1β. A pox virus protein, similar in structure to soluble type II IL-1 receptors, bound only IL-1β and not IL-1α. Production of this protein by the virus reduced the cell-

	IL-1α	IL-1β	IL-1RA
Major Cell Source	Macrophage	Macrophage	Macrophage
Biosynthesis	31 KDa Cytoplasm	31 KDa Cytoplasm	18-22 KDa Golgi-CHO
Proteolytic Processing for Activation	–	+ (17.5 KDa) Asp^{116}-Ala^{117}	–
Receptor Active Form	31 KDa Agonist	17.5 KDa Agonist	22 KDa Antagonist
Secreted *in vitro*	–	+	+
Presence in Disease States (CSF, Synovial Fluid)	+	+++	++++

FIGURE 1. Characteristics of the IL-1 family of molecules binding to IL-1 receptors.

mediated immune response induced by the infection, suggesting that the host response was primarily IL-1β mediated.[26,27]

ROLE OF ICE IN IL-1 ACTIVATION

While both IL-1β and IL-1α are synthesized as 31-kDa forms, only IL-1α is active on IL-1 receptors without further processing; IL-1β must first be processed from its inactive 31-kDa cytoplasmic precursor form (pIL-1β) to an active 17.5-kDa mature form (mIL-1β).[9,17,28] A unique cytoplasmic enzyme thus far found only in monocytic cells has been identified, termed IL-1β converting enzyme (ICE).[29-34] ICE cleaves the Asp^{116}-Ala^{117} bond of pIL-1β to generate the mIL-1β (see FIGURE 2). It also cleaves pIL-1β at a secondary cleavage site Asp^{27}-Gly^{28} to form small amounts of a 28-kDa fragment which can be further processed to the 17.5-kDa form. In contrast, ICE does not appear to cleave other proteins containing Asp-X linkages.[30] ICE is essential for the generation of mIL-1β: cells lacking ICE activity even when transfected with pIL-1β do not form active mIL-1β.[35,36] Another protein, crmA, synthesized by pox viruses provides additional support for the intracellular role of ICE in mIL-1β formation: this serpin inhibits the processing of pIL-1β by ICE.[37] No enzyme has been found to specifically process IL-1α, although calpain has been shown to cleave IL-1α.[38] Because IL-1α remains largely cell associated and is not normally secreted, its appearance on the outside of cells may be associated with cell death.[13,39] The presence of a processed extracellular active 17-KDa form of IL-1α may result from cleavage by other proteases ("bystander proteases") at, for example, the Phe^{118}-Leu^{119} bond[1,40] (see also Reference 41).

No mIL-1β is found inside cells, and little pIL-1β is found outside cells in the absence of cell damage. Pulse-chase analysis indicates that there is a precursor-product relationship between intracellular pIL-1β and secreted mIL-1β.[13,42] Thus, the cleavage of pIL-1β must be closely associated with the secretion of mIL-1β. The unusual mechanism of synthesis, posttranslational modification, and cellular export of IL-1β presents a number of potential sites for therapeutic interdiction. Because of the substrate specificity of ICE, development of an inhibitor for ICE represents a unique opportunity to develop a small molecule inhibitor of mIL-1β formation.

CHARACTERIZATION OF ICE ENZYMATIC ACTIVITY

To determine the minimum recognition sequence of ICE, a 14-amino-acid peptide spanning the Asp-Ala cleavage site of pIL-1β, NEAYVHDAPVRSLN, as well as series of amino-terminal or carboxy-terminal truncations were prepared. These peptides were used as substrates for ICE, and their relative activity (V_{max}/K_m) was compared[34] (FIGURE 3, top). The results indicated that residues beyond P1′ were not required, and at P1′ only a methylamine substituent was necessary. At least four residues to the left of the cleavage site were necessary for activity; no cleavage activity was observed when the Tyr was removed.

Using a pentapeptide to further characterize the relative activity of individual amino acids in the sequence, it was determined that Ac-Tyr-Val-Ala-Asp-Gly was recognized best by ICE (FIGURE 3, bottom). Asp is absolutely required at the P1 position; glutamate at this position is cleaved only slightly. Small aliphatic residues (Gly and Ala) are preferred in P1′. Substitutions in P2 are well tolerated, Val is preferred in P3, and hydrophobic residues are preferred in P4. To facilitate rapid, sensitive measurement of ICE, a fluorometric assay utilizing Ac-Tyr-Val-Ala-Asp-amino-4-methylcoumarin (AcYVAD-AMC) as a substrate was subsequently employed.[34]

ICE was found to be a thiol protease based upon its inhibition by a number of thiol selective reagents such as *N*-ethyl maleimide and iodoacetic acid (TABLE 1). It was not inhibited by inhibitors of serine or aspartyl proteases such as PMSF, leupeptin, or pepstatin. While ICE activity was not inhibited by EDTA, addition of *o*-phenanthroline resulted in inhibition after a prolonged incubation, suggesting that ICE might be a metalloprotease. This inhibition was reversed by high (10 mM) but not low (0.1 mM) DTT. The addition of copper increased the rate of inhibition, and

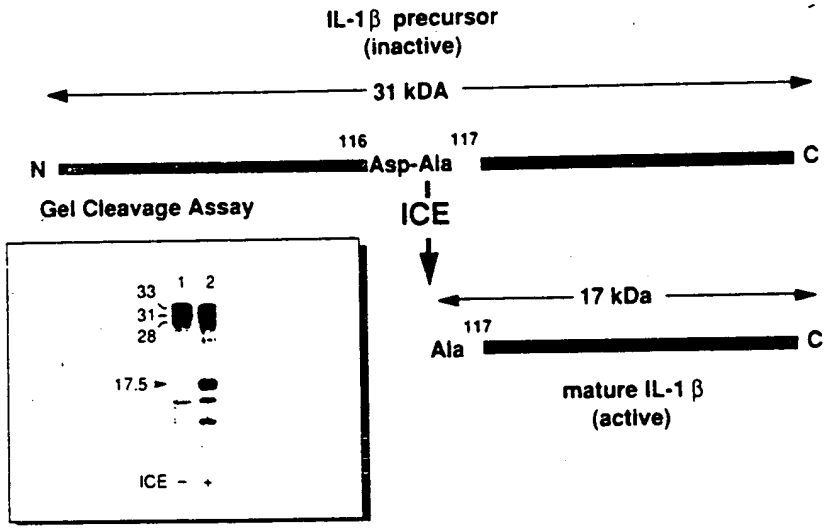

FIGURE 2. Cleavage of the IL-1β precursor by ICE to generate mIL-1β. Inset, autoradiograph of an SDS-PAGE gel of 31-kDa ^{35}S-Met *in vitro* translated pIL-1β cleaved by ICE to 17.5-kDa mIL-1β.[30]

the simultaneous addition of EDTA prevented any inhibition. These results suggested that the *o*-phenanthroline inhibition of ICE occurred by a metal-catalyzed oxidation of a labile thiol.[34]

Definitive evidence that ICE was a cysteine protease came from potent inhibition by a peptide diazomethylketone (L-707,509) and a peptide aldehyde[34] (L-709,049; FIGURE 4). Addition of 250 nM of a peptide diazomethylketone resulted in time-dependent and complete inhibition of ICE activity, but this was prevented by saturating levels of the AcYVAD-AMC substrate (70 × K_m; K_m = 14 μM). Addition of a high concentration of the substrate after inhibition had occurred did not relieve the inhibition, indicating that the inhibition was irreversible. The peptide aldehyde

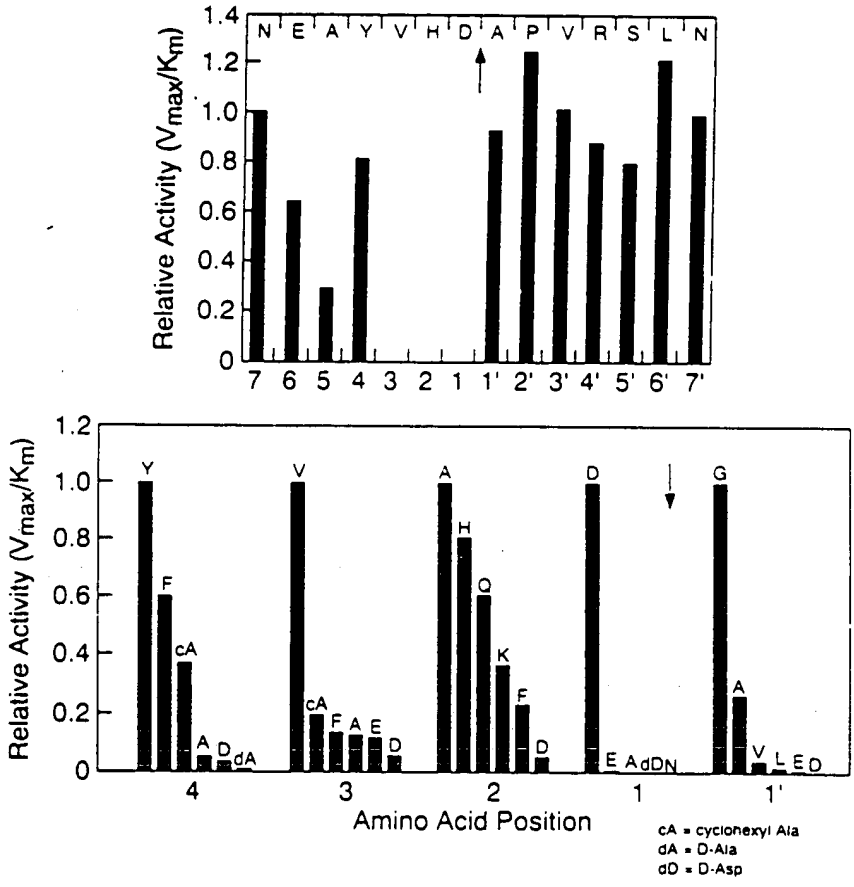

FIGURE 3. Effect of truncation and amino acid substitution on ICE activity. Substrates and products were separated by RP-HPLC and quantitated. Top, ICE activity (V_{max}/K_m) of either N- or C-terminally truncated peptides of the 14-mer pIL-1β spanning peptide NEAYVH-DAPVRSLN; activity was expressed relative to that of the 14-mer defined as 1.0. Bottom, ICE activity of a pentapeptide with the indicated amino acid substitutions; activity was expressed relative to Ac-YVADG defined as 1.0.

TABLE 1. Inhibition of YVAD-AMC Cleavage by Inhibitors of Various Classes of Proteases[a]

Class	Reagent	Inhibition
Serine	PMSF (1 mM)	0 ± 2
	DFP (1 mM)	6 ± 8
	leupeptin (1 mM)	1 ± 2
Aspartyl	Pepstatin (0.1 mM)	0 ± 2
Thiol	NEM (1 mM)	99 ± 1
	Iodoacetate (1 mM)	99 ± 5
	E-64 (1 mM)	0 ± 1
Metallo	EDTA (10 mM)	13 ± 10
	OPA[b] (1 mM) $t_{1/2}$ = 60 min)	98 ± 3
	OPA (1 mM) + 10 μM Cu^{2+} ($t_{1/2}$ = 1 min)	99 ± 3
	OPA + EDTA	10 ± 10
	OPA + 10 mM DTT	10 ± 10
	OPA + 0.1 mM DTT	98 ± 3

[a]Assays were done at the indicated concentration of inhibitors and expressed as a percentage of uninhibited activity. Inhibitors included phenylmethylsulfonylfluoride (PMSF), diisopropylfluorophosphate (DFP), *N*-ethyl maleimide (NEM, and 1,10 (*ortho*)-phenanthroline (OPA).
[b]O-Phenanthroline inhibits ICE via metal catalyzed oxidation.

L-709,049 was also a competitive inhibitor of ICE activity, but saturating levels of substrate added after inhibition had occurred could reverse the inhibition. A competitive substrate peptide hydroxylamine (L-700,018) could also inhibit ICE cleavage, as shown here using *in vitro* translated ^{35}S-Met-labeled pIL-1β (FIGURE 5). ICE was not inhibited by a specific elastase inhibitor (L-680,833).[51]

PURIFICATION AND STRUCTURE OF ACTIVE ICE

Active ICE was purified to homogeneity by conventional ion exchange and reverse phase high-performance liquid chromatography (RP-HPLC) techniques[43] as well as with an inhibitor affinity column.[34] Because the P2 position in ICE substrates was relatively insensitive to substitution (FIGURE 3), a reversible peptide aldehyde inhibitor was prepared with Lys in place of Ala in P2. The Ac-YVAD-CHO was coupled via a spacer arm to Sepharose 4B (FIGURE 6) to generate a specific affinity matrix. Crude dialyzed THP.1 cytosol or a partially purified DEAE pool of that cytosol was allowed to bind to the column and extensively washed. ICE was specifically eluted with 100 μM L-709,049, and found to contain by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) two tightly associated proteins at 20 and 10 KDa (termed p20 and p10 respectively) in a 1:1 ratio (FIGURE 6). To recover active ICE, the L-709,049 bound ICE was first incubated with oxidized glutathione (to form a stable, inactive enzyme-glutathione conjugate) and hydroxylamine (to destroy the aldehyde inhibitor). Secondly, after desalting, DTT was added to remove the glutathione and generate the active enzyme.

Because the ICE active site thiol is more than 10-fold more reactive than ordinary thiols, the Cys could be readily labeled with ^{14}C-iodoacetate. Since the alkylation was competitive with substrate, saturating levels of substrate could prevent the labeling. As shown in FIGURE 7A, ICE inhibition could be almost totally achieved with 100

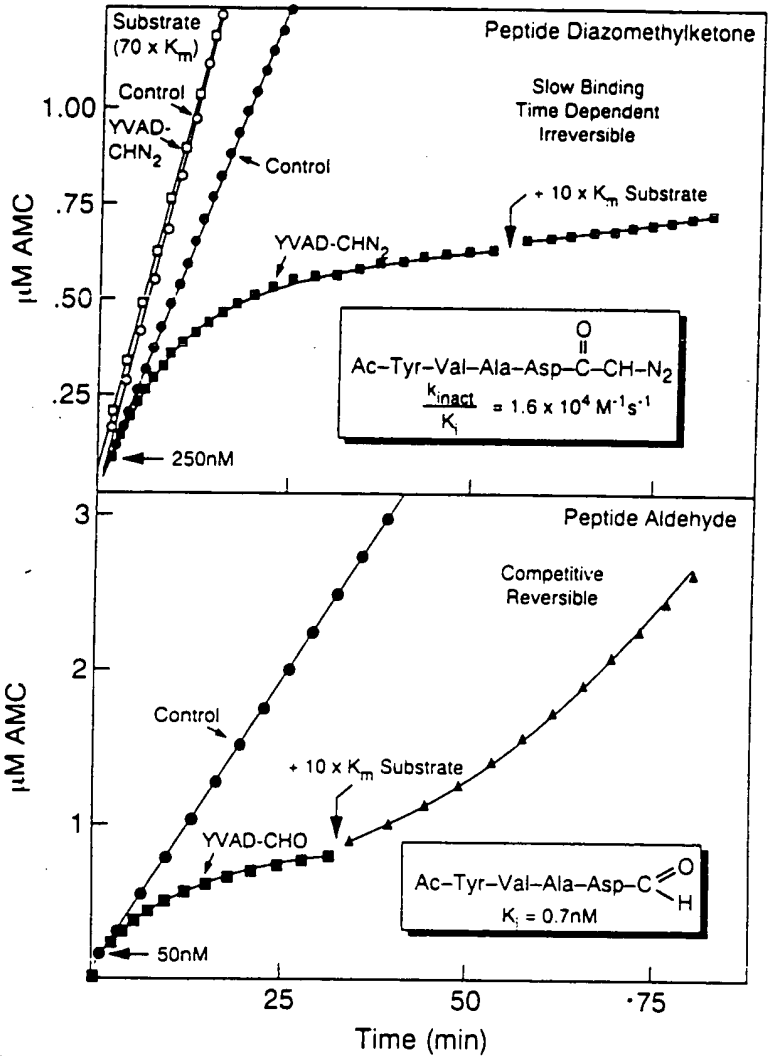

FIGURE 4. Inhibition of ICE by the thiol protease inhibitors Ac-YVAD-diazomethyketone (L-707,509, top) and aldehyde (L-709,049, bottom) using AcYVAD-AMC at its K_m (14 μM). (Taken from Thornberry *et al.*[34] with permission.)

μM iodoacetic acid at 40 min. Under these conditions in the absence of substrate, the p20 protein was selectively labeled (FIGURE 7B). With saturating amounts of substrate where no enzyme inhibition was observed, no p20 labeling occurred. Isolation of the p20 followed by tryptic cleavage and C_8 RP-HPLC separation of the resultant peptides led to the identification of a single labeled peptide (FIGURE 7C) which had the sequence Val-Ile-Ile-Ile-Gln-Ala-(^{14}C)Cys.

After obtaining sequence of tryptic and Asp.N peptides of the p20 and p10

proteins, degenerate oligonucleotides were used to PCR ICE cDNA fragments. These were used t screen a THP.1 monocytic cell cDNA library for full-length cDNA clones. All clones cross-hybridized with probes to both p20 and p10, indicating that both ICE proteins were encoded on a single mRNA. The resultant open reading frame encoded a 45-kDa protein (p45; FIGURE 8) which contained a 13-kDa polypeptide N-terminal to the p20 and a 2-kDa peptide separating the p20 from the p10 which were not found on the isolated active enzyme (FIGURE 9). No sequence homology to other Cys proteases or other proteins in the protein databank was observed.[34] The human cDNA was used to clone out the mouse[44,45] and rat[46] forms of the enzyme (FIGURE 8). All three proteins contained the active site Cys with considerable stretches of amino acid identity particularly in the p10 region where there was 81% amino acid identify between the mouse and human proteins. Identity was less in the p20 region (62%) and still less in the pro domain (53%).[44] An alternatively cleaved, 1.6-kDa higher molecular weight form of the p20, termed p22, was in some cases purified along with the p20 protein (FIGURE 9).[43] All four cleavage sites generating the p20, p22, and p10 proteins followed Asp residues, perhaps indicating autoprocessing of the p45.[34]

The p45 form of ICE is the major form of ICE found in monocytic cells as determined by immunoblots with antibodies generated to affinity purified human ICE. Extracts of p45 have, however, no detectable pIL-1β cleavage activity. Only after dialysis or short incubations at 30°C, when p45 is cleaved to the p20/p10 form of the enzyme, is significant pIL-1β cleavage activity seen.[47] Both p20 and p10 are necessary for ICE activity; ion exchange column fractions of cytoplasmic extracts containing p20 in the absence of p10 contain no ICE activity.[43]

Expression in COS-7 cells of the p45 ICE protein resulted in generation of pIL-1β cleavage activity that could be inhibited by the peptide aldehyde inhibitor L-709,049 (FIGURE 10). An ^{116}Asp to Ala mutant of pIL-1β was not a substrate for

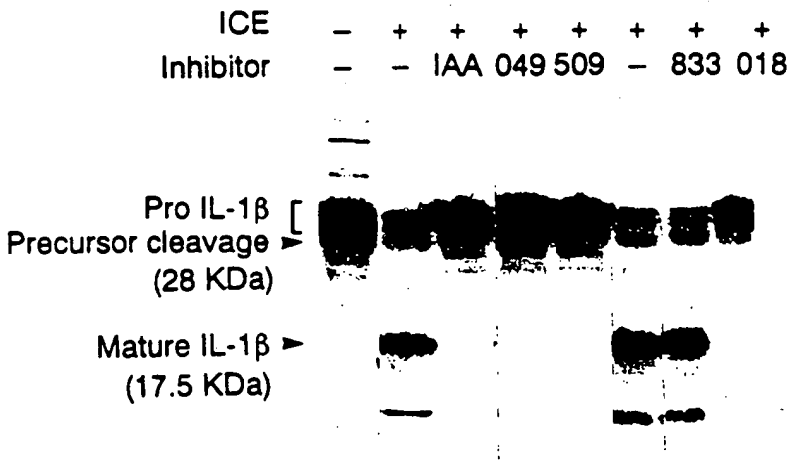

FIGURE 5. Inhibition of ICE cleavage of ^{35}S-Met-labeled pIL-1β by various inhibitors. **IAA**, iodoacetic acid, 5 mM; **049**, L-709,049, AcYVAD-CHO, 0.5 μM; **509**, L-707,509, AcYVAD-CN_2, 10 μM; **833**, L-680,833[51], 5 μM; **018**, L-700,018, AcYVAD-NHOH, 0.5 mM.

the recombinant ICE.[34] Expression of human p45 in *Escherichea coli* or *baculovirus* systems, followed by purification on the peptide aldehyde affinity column resulted in the purification of both p20 and p10 in 1:1 ratios.[48] Presumably the N-terminal domain of the p45 is necessary for proper folding of the p20 and p10 proteins;

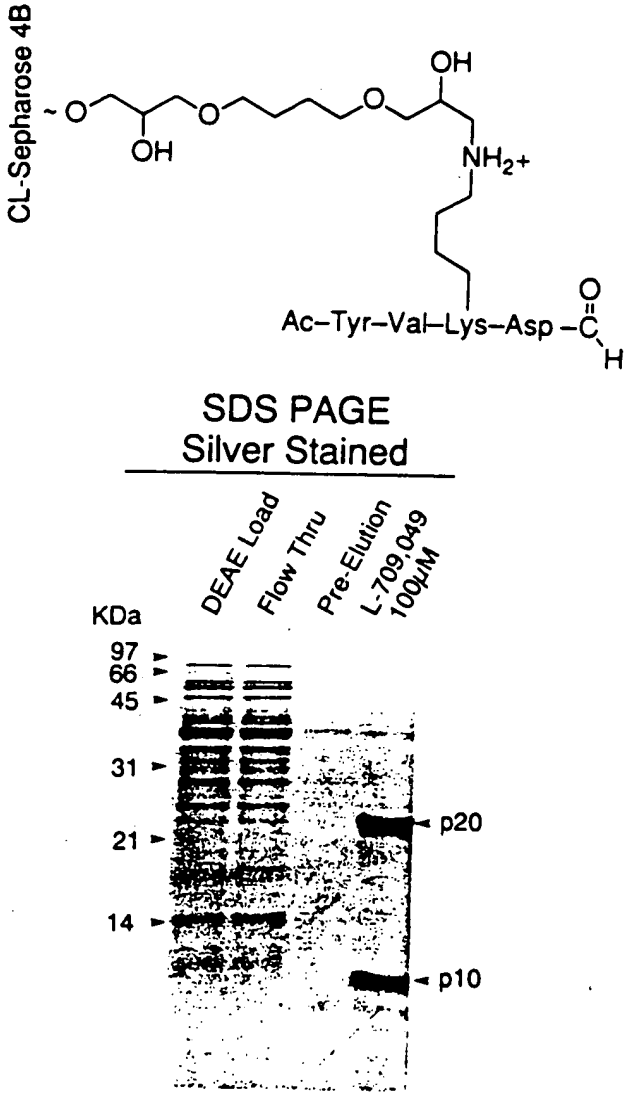

FIGURE 6. Affinity purification of ICE. **Top:** A peptide aldehyde affinity matrix was prepared by coupling AcYVKD-CHO to Sepharose 4B as shown.[34] **Bottom:** Silver-stained SDS-PAGE of fractions from an affinity column purification beginning with a partially purified DEAE fraction of ICE.[43] The load, the flowthrough fraction, a column fraction after extensive washing, and the proteins eluted by 100 μM L-709,049 are shown.

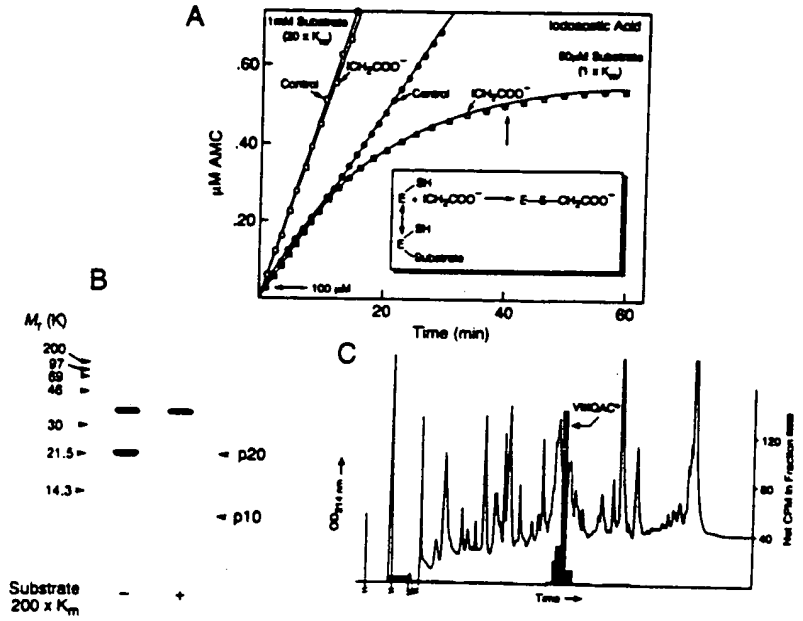

FIGURE 7. Inhibition of ICE by ^{14}C-iodoacetic acid and its labeling of the active site Cys. A: Kinetic analysis of iodoacetic acid inhibition of ICE using 100 μM iodoacetic acid and its competition by saturating substrate. B: Under conditions of 99% inhibition of ICE (40 min), the p20 protein found in a partially purified sulfopropyl-HPLC fraction of active[43] THP.1 ICE was labeled and identified following SDS-PAGE and autoradiography.[34] C: C_8-RP-HPLC chromatography of the tryptic peptides of the p20 labeled with ^{14}C-iodoacetic acid (the p20 was purified by C_4-RP-HPLC prior to trypsin cleavage). Only one peptide was labeled, and sequencing of this peptide revealed that the active site Cys was found in a sequence VIIIQAC.[43]

coexpression of isolated p20 and p10 together in *E. coli* did not produce any active enzyme.[48] The activity of the recombinant enzyme was comparable to that of the native THP.1 enzyme: the ICE inhibitor affinity column yielded similar amounts of isolated enzyme for the same amount of activity units applied. Occasionally a processed form of p10, termed p7, presumably also formed by autocatalysis, could be copurified on the affinity column.[48] This form could also be generated in small amounts in highly purified fractions of THP.1 ICE where it contained substantially less activity.[43]

EFFECT OF INHIBITION OF ICE ON IL-1β SECRETION FROM MONOCYTES

To determine the effect of ICE inhibitors on mIL-1β production in human monocytes, ^{35}S-Met labeled heparinized blood was stimulated with heat-killed *Staphylococcus aureus* which has been shown to promote both rapid synthesize of pIL-1β as well as rapid release extracellularly of mIL-1β.[49] When this stimulation was performed on cells preincubated with the peptide aldehyde inhibitor L-709,049, mIL-1β release was inhibited in a dose-responsive fashion with an IC_{50} of about 2

μM (FIGURE 11). In contrast, the addition of a control peptide aldehyde with a D-Ala residue in the P3 position (K_i = 1.5 μM vs. K_i = 0.8 nM for L-709,049) resulted in little observable inhibition of mIL-1β release.[34] The specificity of this inhibition for IL-1β release was shown by the lack of inhibition of TNFα, IL-6, or IL-8 release by the same cells (FIGURE 12).

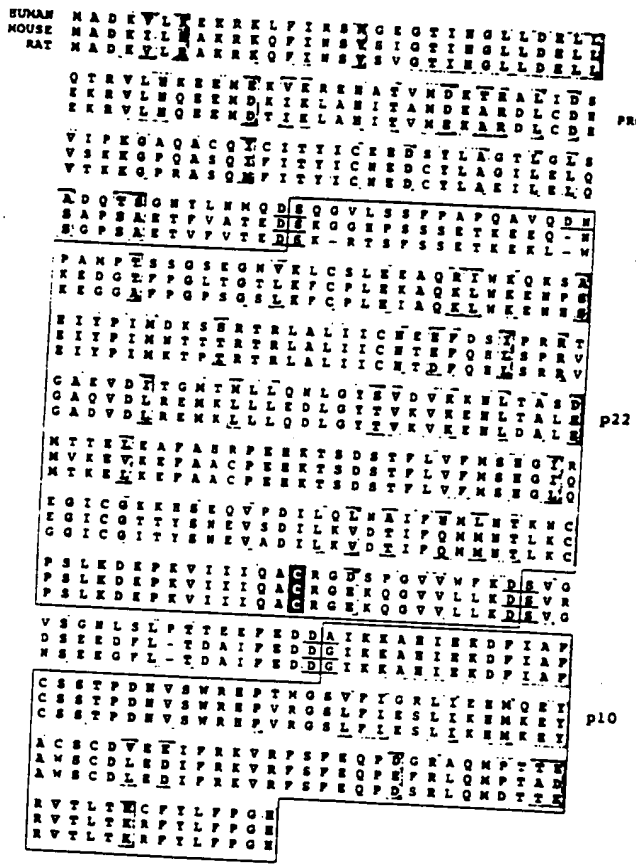

FIGURE 8. The amino acid sequence of human, mouse, and rat ICE. ICE was cloned as described,[34,44,46] and the aligned open reading frame is shown. Shaded areas indicate amino acids of identity or close similarity. The boxed in regions correspond to the sequence contained within the p22 and p10 proteins. The active site Cys is shown in black. Sites of cleavage to form the individual p22, p20, and p10 proteins are underlined.

DISCUSSION

We have succeeded in purifying, characterizing, and expressing ICE, a unique Cys protease from monocytic cells that cleaves pIL-1β at the Asp^{116}-Ala^{117} bond. That ICE is a cysteine protease is shown by its sensitivity to known nonspecific thiol

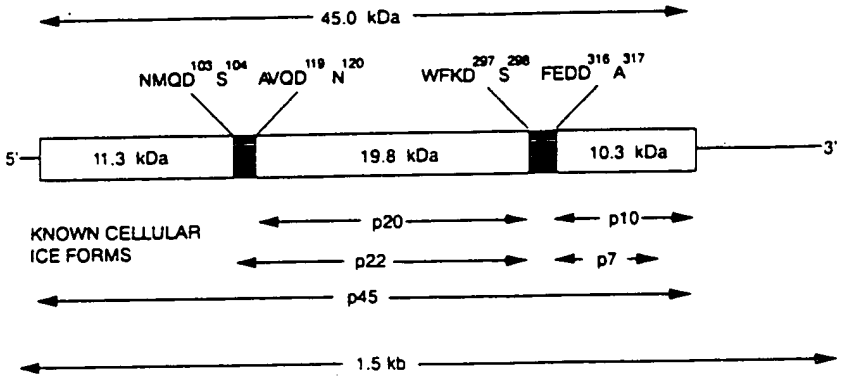

FIGURE 9. Structural organization of the human ICE precursor protein (p45) and the sites of cleavage found in ICE forms identified in THP.1 cells and recombinant expression systems, including p22, p20, p10, and p7, a truncated form of p10.

alkylating agents such as NEM and iodoacetic acid, and its insensitivity to Ser, Asp, or metalloprotease inhibitors. More definitively, such specific agents as tetrapeptide aldehydes or diazomethylketones inhibit ICE, while, in contrast, a truncated carbobenzyloxy aspartyl diazomethylketone is 10,000-fold less potent as an ICE inhibitor.[34] Furthermore, the replacement of the active site Cys with Ala totally eliminates any ICE activity.[50] The minimum recognition sequence for ICE is a relatively small

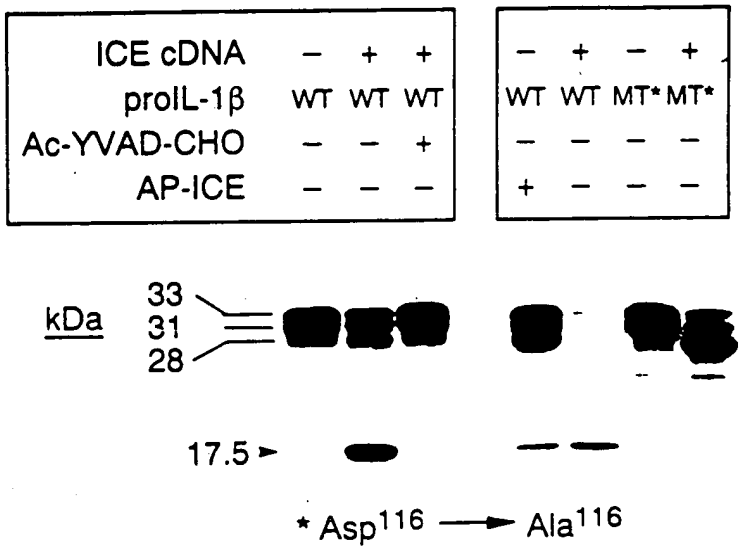

FIGURE 10. Functional expression of active recombinant ICE expressed in COS-7 cells. Extracts of cells transfected with human p45 ICE were incubated with ^{35}S-Met-labeled wild-type pIL-1β (WT) or a Ala^{116} mutant of IL-1β (MT) in the presence or absence of affinity purified ICE (taken from Reference 34 with permission). The resultant samples were separated by SDS-PAGE and visualized by autoradiography.

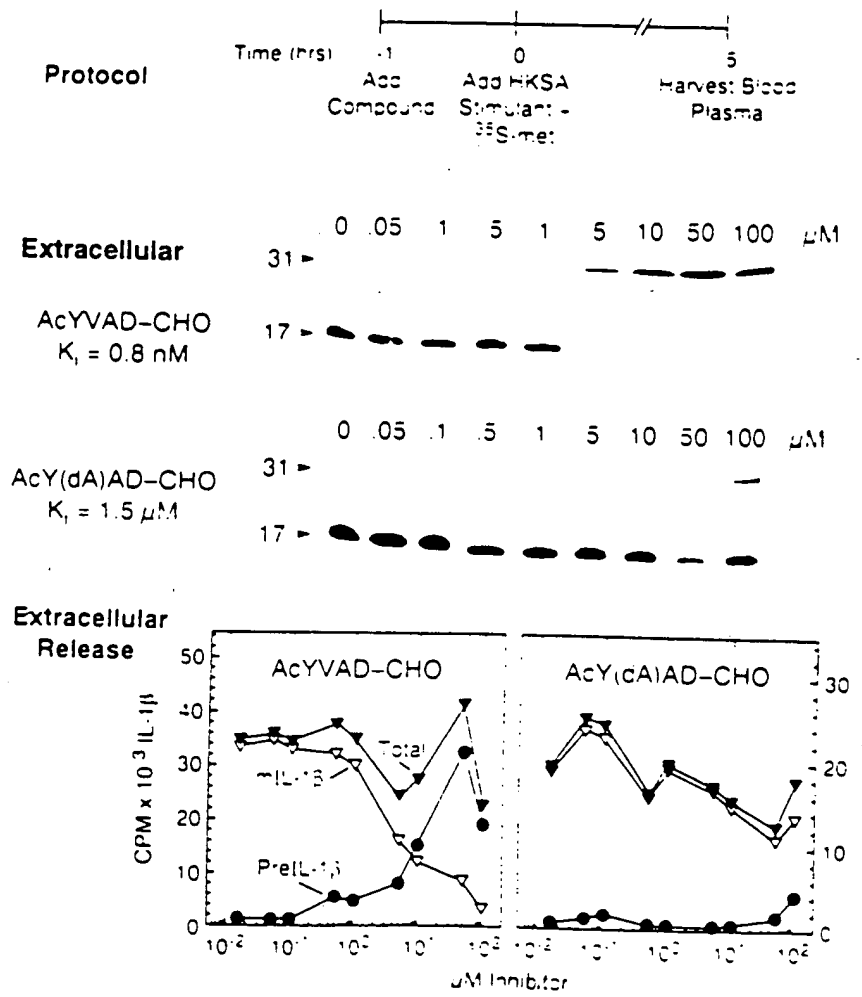

FIGURE 11. Inhibition of mIL-1β release from human peripheral blood monocytes by a peptide aldehyde inhibitor (Ac-YVAD-CHO) or a relatively inactive D-Ala control peptide (AcYdAAD-CHO). ^{35}S-Met-labeled heparinized blood was preincubated with indicated concentrations of the inhibitor for 1 h, followed by a 5-h incubation with heat-killed *Staphylococcus aureus*. The plasma was removed and immunoprecipitated with antiIL-1β antiserum, and the immunoprecipitates were separated by SDS-PAGE and subjected to autoradiography. Radioimaging of the individual bands is shown at the bottom (Taken from Reference 34 with permission).

tetrapeptide characterized most prominently by the absolute necessity for an Asp in P1 and secondarily by the need for a relatively large hydrophobic group in P4. ICE bears no homology to other known Cys or Ser proteases; the original observation of active site sequence similarity to Ser proteases[34] was not borne out when the mouse and rat sequences were obtained[44–46] in that the Ser289 was replaced by a Lys.

ICE itself appears to require processing before it can become active. While it is synthesized as a 45-kDa protein and is the predominant cellular form seen in monocytic cells, it has no detectable pIL-1β cleavage activity until removal of a precursor domain of about 13 kDa and a 2-kDa intervening piece between the p20 and p10 proteins. Exactly how and where this processing occurs is not known, but all of the cleavage sites are preceded by Asp residues. Because purified ICE can cleave the p45 precursor, it is possible that this processing is autocatalytic.[34]

Whereas active ICE is a complex of freely dissociable inactive monomers,[34] there is no evidence that the p20 and p10 polypeptides themselves are freely dissociable from one another. Simultaneous expression of both p10 and p20 in *E. coli* does not generate active ICE.[48] Furthermore, purification by ion exchange columns of active ICE from THP.1 cytoplasmic extracts has shown no evidence for p10 separate from p20.[43] What is seen is that p10 is susceptible to proteolysis, and that p20 is found associated not only with intact p10, but also with a lower molecular weight C-terminally cleaved form of p10 (p7) forming an ICE complex with reduced activity. p20 can also be found with all of the p10 removed, in which case no ICE activity is seen.[43] Thus, the p10 part of ICE is clearly needed for ICE activity. Not surprisingly, it is the most conserved portion of ICE (FIGURE 8).

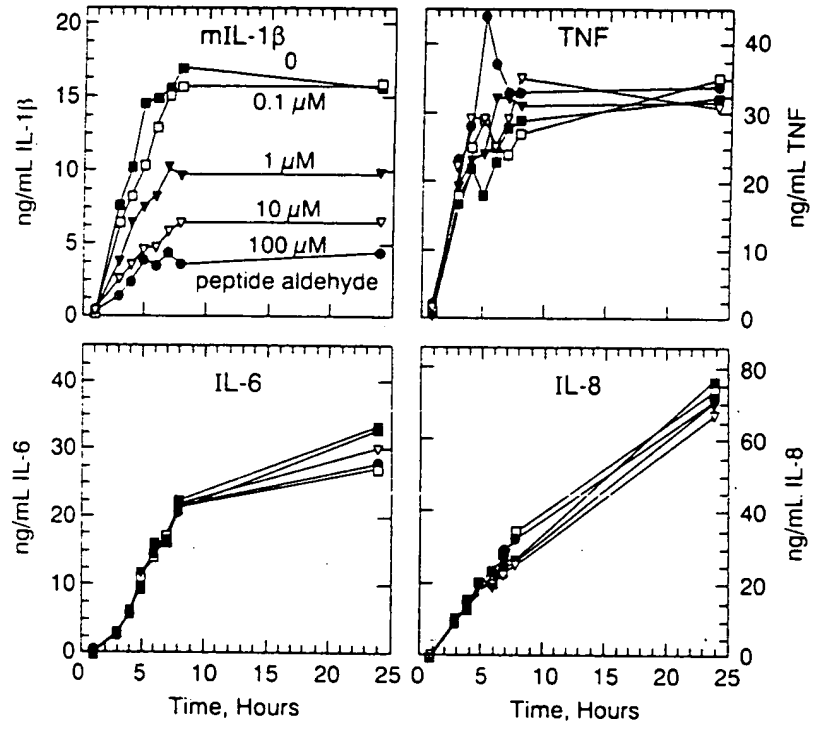

FIGURE 12. Effect of a specific ICE inhibitor on release of cytokines from *S. aureus*–stimulated human peripheral blood monocytes. Various concentrations of the peptide aldehyde inhibitor (AcYVAD-CHO) were preincubated with cells as described in FIGURE 13, and the supernatants were analyzed by ELISA for IL-1β, TNFα, IL-6, or IL-8 release.

We have shown that specific inhibitors of ICE can be synthesized; the peptide aldehyde inhibitor affinity column will purify active ICE from THP.1 cytosol in a single step. Furthermore, this inhibitor can inhibit release of mIL-1β from activated monocytes without preventing the release of TNFα, IL-6, or IL-8. Even though inhibition of ICE inhibits cellular pIL-1β processing, pIL-1β secretion from the monocytic cell is unaffected (see FIGURE 13); that is, secretion occurs independently of processing. A critical issue, then, in the development of a therapeutic ICE inhibitor is whether or not pIL-1β might be processed by "bystander proteases" at sites of inflammation to yield an active product with activity similar to mIL-1β. Secondly, there is the question of whether other cytokines such as TNFα or IL-1α released from damaged cells might be sufficient to maintain the inflammatory response.

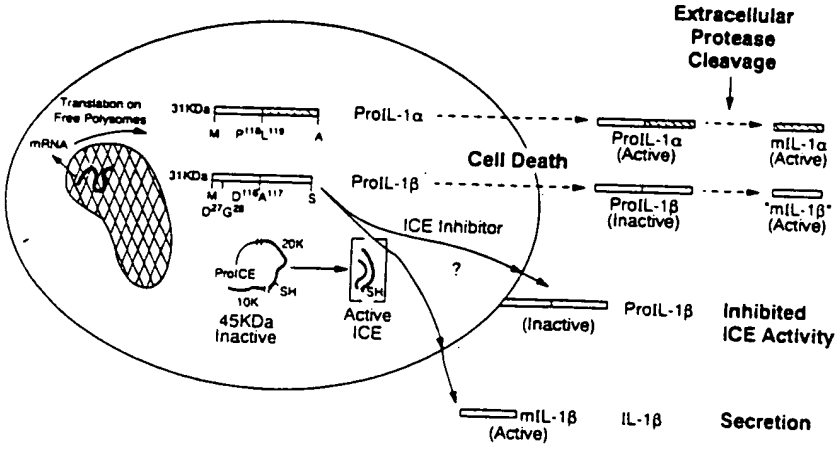

FIGURE 13. Schema of IL-1 processing and secretion. IL-1α and IL-1β are synthesized as 31-kDa cytoplasmic precursor proteins. The precursors are ordinarily not released from cells except following cell damage or death, at which time they could be potentially cleaved by other proteases. The 31-kDa IL-1β is normally processed to the 17.5-kDa mIL-1β by an active form of ICE that is itself activated from a 45-kDa precursor. Cleavage of pIL-1β occurs independently of mIL-1β secretion, since inhibition of ICE cleavage results in extracellular accumulation of pIL-1β.

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